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Review

Gene transfer in higher animals: theoretical considerations and key concepts

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Abstract

Gene transfer technology provides the ability to genetically manipulate the cells of higher animals. Gene transfer permits both germline and somatic alterations. Such genetic manipulation is the basis for animal transgenesis goals and gene therapy attempts. Improvements in gene transfer are required in terms of transgene design to permit gene targeting, and in terms of transfection approaches to allow improved transgene uptake efficiencies. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

During the 1970s it became possible to introduce exogenous DNA constructs into higher eukaryotic cells in vitro. Mammalian (germline) transgenesis was first achieved in the early 1980s, mice being the subject species. Transgenic members of a wide range of animal (and plant) classes and species have now been produced, including amphibians, cattle, chickens, fish, insects, nematodes, pigs, rabbits, and sea urchins.

Gene transfer methods have been used in gene therapy attempts on humans since 1990. Gene therapy approaches have so far focused primarily on monogenic disorders and cancers. To date, limited clinical success has been achieved. However, gene therapy is in its infancy and holds great promise for the future.

As indicated above, gene transfer methods may be used to generate transgenic animals. Such animals may in principle be utilised in either of two broad ways: (a) as models for fundamental or applied scientific study; and (b) as novel sources of pharmaceutical agents, or human-compatible organs for xenotransplantation.

Gene transfer in higher eukaryotes may in principle be applied directly for therapeutic purpose to humans in either of two broad ways: (a) somatic gene therapy, the genetic manipulation of a subset of cells in the body; and (b) germline gene therapy, involving an alteration of genetic information in germ cells. Germline gene therapy has never (yet) been attempted with humans (unless one includes the transfer of foreign mitochondria during fertilisation), and is fraught with major

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ethical concerns, thus there is a dearth of scientific literature available on the subject.

2. Cell types

To achieve germline alterations, transgenesis must occur at an early stage of development. By contrast, somatic cell alterations, as in somatic gene therapy, involve a very wide range of cell types. Various somatic cell types are mentioned at appropriate points throughout this paper. The present section reviews the cell types that may be used for germline transgenesis.

2.1. Egg cells

Genetic manipulation of a newly fertilised, single-cell egg (zygote) should in principle result in the development of an organism in which all or very many cells contain the (identical) alteration. Hence, the zygote has been the main focus for transgenic engineering. (In keeping with common usage, in this paper the term 'egg' is applied to all developmental stages from oocyte to unhatched blastocyst.)

Pre-fertilisation eggs may in principle be suitable targets for transgenesis. However, fundamental practical problems have so far precluded their use. Eggs collected following ovulation would have to be fertilised after transgenic manipulation. This would entail the use of in vitro fertilisation. Potentially-transgenic eggs would thus have to endure a further, extensive ex vivo procedure. Since this could only be detrimental to the eggs, it is difficult to see any role for transgenesis directed at this level rather than at the zygote.

Speculatively, en masse transgenic manipulation of pre-ovulatory oocytes in vivo could in principle be attempted. The resultant 'proto-transgenic' organism would be expected, upon each subsequent ovulation, to produce transgenically altered eggs ready for fertilisation. However, the relevant technology has not yet been sufficiently developed to support this type of in vivo approach, and no transgenic animals are reported to have been generated in this way.

Eggs that have undergone cleavage are less than ideal for transgenesis. Where only one cell is transgenically altered, the resulting organism is likely to take the form of a genetic mosaic. Such an organism would consist of: (a) cells harbouring the transgene; and (b) cells without the transgene. Indeed, the more rounds of cleavage there have been prior to transgenesis, the lower the probable proportion of altered cells in the transgenic. Manipulating more than one cell in an egg is technically very difficult. More crucially, each manipulated cell would not contain an identical alteration, for reasons to be explored later. Thus, although comprising only altered cells, the resultant organism would again be a mosaic (Jaenisch, 1988; Whitelaw et al., 1993; Wilkie et al., 1986).

Mosaicism need not always be a problem. Mosaic transgenics may produce gametes that contain the transgene, allowing the subsequent generation(s) to be fully transgenic. However, mosaic transgenics do not always contain genetically altered germline cells. Of those transgenics that do, not all of the germline cells will necessarily be altered. Thus the use of post-zygotic eggs would result in a lower ultimate efficiency of transgenesis in comparison with zygote-stage eggs.

A major limitation associated with eggs is that the targeting of transgenes to chosen genomic loci is not a practical proposition, due to the inability to select for eggs that contain rare targeted integration outcomes. Although gene targeting has been reported following gene transfer to eggs, the rate of targeting (versus random integration) is too low for practical purposes (Brinster et al., 1989). However, many transgenic experiments do not require gene targeting (Rulicke and Hubscher, 2000). Randomly integrated transgenes have been used in a variety of approaches. Examples include controlled trangene expression via administration of an extrinsic agent (Kistner et al., 1996), ablation of hormone-producing tissues by expression of a toxin-producing transgene (Wallace et al., 1991) and production of human blood clotting factor VIII in the milk of transgenic sheep (Niemann et al., 1999).

2.2. Embryonic stem cells

Inner cell mass (ICM) cells from the mouse blastocyst can be propagated in vitro as embryonic stem (ES) cells (reviewed by Torres, 1998). In contrast to other cultured cell lines, ES cells retain their normal karyotype even after several weeks in culture, during which time they remain totipotent. Furthermore, ES cells are capable of colonising the embryo. These unique properties allow ES cells to form chimeras when injected into blastocysts or aggregated with morulae. The resultant embryos are transferred to the uterus of a pseudopregnant female mouse, where approximately 50% should develop successfully to term. Approximately 50% of the resultant offspring should be chimeric. The ES cell contribution to a mouse can be as high as 80% of the cells, and will often include the germline cells.

It is during the in vitro culture stage that ES cells may be transgenically manipulated (Pirity et al., 1998). The great advantage of ES cells is that they can be subjected to a range of selective agents in vitro, which allows the selection of particular transgenic modifications. This ability makes ES cells extremely useful for gene targeting experiments and applications.

ES cell technology has enabled a large range of transgenic approaches in the mouse. Types of gene modifications presently available in the mouse include targeted elimination of endogenous gene expression (gene 'knockout'), targeted gene repair/replacement, conditional gene targeting and 'gene trap' reporter systems (for relevant reviews see Demayo and Tsai, 2001; Stanford et al., 2001; Gao et al., 1999; Jasin et al., 1996; Ledermann, 2000; Lewandoski, 2001; Metzger and Feil, 1999; Muller, 1999).

The use of ES cells is limited due to the fact that, to date, the mouse is the only animal from which ES cell lines have been unequivocally established. It would be surprising if this limitation represents a fundamental biological barrier. However, further empirical work is needed before true ES cell lines become available for other species. It is possible that the inbred strains of mice used to generate ES cells may carry mutations that are essential for the generation of ES cells. If such mutations represent a precondition for ES cell derivation, then it may take a considerable amount of time to establish nonmurine ES cell lines. Nevertheless, major progress has recently, been made in the analysis of molecular pathways of ICM and trophoblast differentiation in mammals (Niwa, 2001; Rossant, 2001). Such progress is expected to have a positive impact on nonmurine ES cell establishment. When nonmurine ES cells become available, the established mouse technologies will provide the basis for in vitro genetic modification of all species.

2.3. Nuclear transfer possibilities

Building upon fundamental research into cell cycle co-ordination, Wilmut et al. (1997), Schnieke et al. (1997) and Campbell et al. (1996) have reported the successful transfer of 'reprogrammed' ovine donor nuclei. Unfertilised, metaphase-stage enucleated ('universal recipient') eggs received the transferred nuclei. Nuclei were taken from somatic cells that had been forced into a form of cell cycle stasis (by incubating the cells in a minimal nutrient medium), such that DNA replication and gene expression were halted (or virtually so). The transfer of 'static' donor nuclei to 'universal recipient' eggs resulted (in some cases) in successful embryo development, the donor nuclei having been 'reprogrammed' into totipotency. Offspring were produced following the transfer of such 'reconstructed' embryos to recipient ewes. Subsequent molecular genetic testing showed that the lambs' DNA had originated from the donor cells. In some of the experiments, the donor nuclei were obtained from (ovine) embryo-derived cultured cell lines. Following these ground-breaking experiments, successful cloning from cultured cells of various animals including cattle, goats and pigs has been reported (see reviews by Tsunoda and Kato, 2000; Wolf et al., 2000).

The prospects for germline transgenesis via nuclear transfer (NT) are very significant: transgenes can be introduced to donor cells in vitro, permitting the production of genetically modified animals by NT (Schnieke et al., 1997). Moreover, because selection can be applied to cultured donor cells, NT can be used to produce gene-targeted transgenic animals (reviewed by Clark et al., 2000).

The generation of the first gene-targeted sheep by Mccreath et al. (2000) provides a useful illustration. In this approach, fetal fibroblasts were transfected with a therapeutic transgene carried by liposomes (see Section 3.3.3). Key parts of the transgene construct included (a) sequences homologous to the ovine α1(I) procollagen locus and (b) a promoterless *neomycin* selectable marker. These transgene features were employed such that (a) homologous recombination (HR) between transgene and target would result in targeted transgene integration, and (b) targeted integration would confer G418 resistance by bringing the *neomycin* gene into proximity with the endogenous promoter. G418-resistant fibroblasts were cultured in reduced serum medium prior to the transfer of their nuclei into recipient (enucleated) oocytes. From 470 reconstructed embryos, 20 fetuses were produced, from which 14 live-born lambs resulted. Of 16 lambs and fetuses analysed, 15 showed the presence of the transgene at the target locus.

Similarly, Lai et al. (2002) have recently reported successful NT gene targeting in pigs. The target was the alpha-1,3-galactosyltransferase locus in porcine fetal fibroblasts. Following in vitro selection, 338 reconstructed embryos were created, which in turn gave rise to 7 live-born piglets. Of these, 4 piglets contained transgene DNA at the target locus, such that in each case one allele of the alpha-1,3-galactosyltransferase gene was knocked-out.

Thus, NT is potentially able to provide the same range of transgenic manipulations presently available in mice (via the ES cell route) to all animal species. However, in comparison with ES cell transgenesis, NT has thus far proved to be relatively inefficient: only a small proportion of reconstructed embryos survive to become live animals. For example, in the foregoing cases, live targeted sheep were produced at an efficiency of 3.6% (Mccreath et al., 2000), and pigs at 1.2% (Lai et al., 2002).

The health status of NT-derived animals is also proving to be problematic (reviewed by Smith et al., 2000; Renard et al., 2002). Developmental abnormalities are very common, and frequently result in death (fetal or postnatal) or debility. For example, of the 14 live-born lambs described above (Mccreath et al., 2000), seven died within 30 h of birth, and four died within 12 weeks. Similarly, out of the 7 piglets described above (Lai et al., 2002), 2 piglets died shortly after birth, and one died at 17 days; only one appeared to be entirely free of developmental abnormalities. Transgenesis and gene targeting are not of themselves implicated: the health problems are associated with NT per se. During the in vitro (cell culture) stage, the pattern of chromosomal imprinting may change; there are indications that inappropriate expression of imprinted genes following such epigenetic alteration may be mainly responsible for the poor health of NT-derived animals (Kono, 1998; Rideout et al., 2001; Wakayama and Yanagimachi, 2001). Research into epigenetic reprogramming in NT embryos is in progress, and it is to be hoped that developmental abnormalities arising from NT will eventually be eliminated or reduced in frequency. Meanwhile, it is anticipated that NT-related health problems, to the extent that the basis for such is epigenetic, are unlikely to affect the offspring of surviving first generation animals.

2.4. Sperm cells

As should become clear later, one of the major technical drawbacks of germline transgenesis is the difficulty of physically introducing exogenous DNA (the transgene) to the zygote. Given that the natural role of sperm cells is to deliver DNA to the egg, an intriguing approach would be to induce sperm cells to carry transgenes. A number of groups have claimed varying degrees of success in this regard. However, others have had difficulty replicating such work, and there are as yet no clear answers to the questions: (a) Is it possible; and (b) If so, how? (reviewed by Smith, 1999).

An alternative possibility could be to introduce the transgene into testicular (sperm) stem cells in vivo. This would in principle remove the need to collect, manipulate or transfer eggs, thus providing a major streamlining of germline transgenesis. Preliminary results have been reported in mice, where transgene constructs were directly injected into the testis. For example, 60–70% of sperm were reported to carry the transgene following

injection of naked DNA into the vas deferens (Huguet and Esponda, 1998), with a follow-up report claiming detection of the transgene in the cells of 7.5% of offspring animals produced following fertilisation with the transgene-bearing sperm (Huguet and Esponda, 2000). Similar results were reported by Sato et al., using lipid-associated transgene molecules injected through the testicular capsule (Sato et al., 1999).

3. Methods for introducing transgenes

This section reviews several methods that may be used for getting exogenous DNA into recipient cells. The most widely used/widely applicable methods—retroviral transfer and micro-injection—are considered first. Some fundamentals of transgene design are also considered, because most work in this regard has probably been carried out (and has the widest scope) with microinjection, at least as far as germline modifications are concerned. However, such fundamentals of transgene design also apply to other delivery methods, as should become clear in later sections of this paper. Other delivery methods—i.e. those currently under development or less widely applicable—are considered also.

3.1. Retroviral infection

Retroviruses are found in many species including most mammals (reviewed by Lazo and Tsichlis, 1990). Retroviruses have RNA as their genetic material. Following infection, the RNA is transcribed by the virus-encoded enzyme reverse transcriptase. The resultant single-stranded DNA is replicated as double-stranded DNA (dsDNA). The dsDNA viral genome has the important property of being able to linearly integrate (as a provirus) into the chromosomal DNA of the host cell. The site of integration appears to be essentially random.

The genome of retroviruses can be manipulated to carry exogenous DNA. Eggs may be incubated in media containing high concentrations of the resultant retroviral vector. Alternatively, retroviral vector-producing cell monolayers may be used, upon which eggs are co-cultivated. In either case, between 10 and 50% of (surviving) embryos will be infected. Following egg transfer into pseudopregnant females, the infected embryos should give rise to transgenic offspring. Molecular genetic analysis of transgenics produced in this way usually show integration of a single proviral copy into a given chromosomal site. Rearrangements of the host genome are normally restricted to short direct repeats at the site of integration. In many embryos the germline cells contain viral integrants: thus, transmission of the transgene to the next generation will often occur. Methods have also been developed to allow infection into postimplantation embryos. Virus uptake is effective for many somatic cell lines, however germline cells are infected at low frequency, due to a high level of mosaicism (Braas et al., 1996; Morgan and French Anderson, 1993).

Infection into highly developed tissues, such as those of foetuses, juveniles or adults, is also a possibility (reviewed by Hu and Pathak, 2000). This holds great promise in the context of somatic gene therapy. Retroviral vectors have also been used to introduce transgenes into the ES cell genome (for example, see Robertson et al., 1986).

The major advantages of retroviral vectors are: (a) is the ease of introducing the transgene (the virus is naturally equipped to carry exogenous genetic material into cells and to integrate it into the cells' endogenous DNA); and (b) the unitary form of integration (one intact copy per genome of transgene-positive cells is the norm). However, retroviral vectors are limited or problematic in a number of respects, as discussed in the following sections.

3.1.1. Cell status

Because the size of the viral DNA–protein complex precludes it from passing through the nuclear pores, the host cell must be in mitosis for integration to occur (Miller et al., 1990). Thus only actively dividing cells are infectable by retroviral vectors. This means that: (a) retrovirus-based gene therapies would not be applicable to many cell/tissue/organ types in the adult (e.g. brain tissue); and (b) eggs need to be at the eight-cell stage (at

least) before infection can begin: this leads to problems of mosaicism.

3.1.2. Size limits

Retroviral vectors are somewhat limited in respect of the length of transgene sequence it is possible for them to carry. The upward length of transgene sequence is 9–10 kb (for example see Hu and Pathak, 2000).

3.1.3. Expression problems

Retroviral vectors integrate into the host DNA in a largely random fashion. Thus the chromosomal location of the provirus/transgene varies between individual transgenics created with an identical retroviral vector construct. Each particular chromosomal environment is likely to have a particular effect on the transcription of the transgene. The result is inconsistency and irreproducibility of expression (for example see Jahner and Jaenisch, 1985).

3.1.4. Control problems

Transgene expression in the provirus is driven by the viral 5′ long direct terminal repeat. Hence, it is problematic to engineer into the construct the ability for it to be controlled (a) in a tissue-specific manner or (b) by external (experimenter) influence (for example see Hoatlin et al., 1995; Pannell and Ellis, 2001).

3.1.5. Low frequency of homologous recombination

Using integration-deficient retroviral vectors, Ellis and Bernstein (1989) were able to target genomic loci such that vector sequences homologously recombined with endogenous sequences. However, the frequency of targeting was very low (≈ 1 targeted event per 3×10^6 infected cells). Given the essentially random (nonhomologous) mode of integration of natural retroviruses, retroviral vectors do not appear to hold much promise for applications in which gene targeting is required.

3.1.6. Instability/safety concerns

It is possible for integrated retroviral DNA to be spontaneously reactivated (Weiss et al., 1985), leading to the production of new integration within the DNA of the cell, to new infection of other cells or to infection of other individual animals.

Such instability again may result in transgene expression problems and safety concerns (reviewed by Temin, 1990; Cornetta et al., 1991; Gunter et al., 1993). However, retroviral vectors may be engineered such that they lack the genetic sequences required for a normal life cycle (reviewed by Vile et al., 1996). The creation of such ‘defective’ retroviral vectors goes a long way towards curing instability-related expression and safety problems. However, the risk of reactivation can probably never be completely eliminated, because complementation by a competent ‘helper’ retrovirus cannot be ruled out. In a controlled laboratory environment this may well represent only a minor concern. However, for transgenically altered organisms entering the outside environment (e.g. transgenic dairy cattle bioreactors, or humans treated with gene therapy), the risk may be more acute. In addition to the risks of releasing infectious agents into the general environment, there are concerns for patients who have been treated with retroviral vector-based gene therapeutic agents. In such cases, retroviral reactivation could conceivably lead to oncogenesis.

Thus there are serious safety concerns over the use of retroviral vectors. Such risks are generally difficult to qualify. Whatever the actual risk may turn out to be, in the absence of certainty most regulatory bodies have tended to be fairly restrictive with regard to what they will allow in respect of retroviral vector usage (for example, see Kessler, 1993).

In fact, a wide range of safety issues surrounds the use of retroviruses as agents of exogenous gene delivery. The following list summarises the main areas of concern.

- *Integration problems:* Accidental integration into or near to an endogenous gene could lead to insertional mutagenesis/oncogene activation (although this is not unique to the retroviral method of gene delivery).
- *Pathogenesis:* Immunocompromised patients may be at risk from infection: such infection could result in direct damage or oncogenesis.

- *Pathogen contamination:* If the packaging cells become infected—especially with another virus—this agent is likely to contaminate the retroviral product.
- *Pseudotyping:* Surface/structural changes to the virus may occur due to packaging cell viral-based sequences: this may result in altered/expanded host cell range (paradoxically, this may be useful if harnessed correctly, in some possible applications—see review by Dornburg, 1997).
- *Complementation:* As alluded to previously, complementation by a competent 'helper' retrovirus cannot be completely ruled out. This may occur in vitro or in vivo.

3.1.7. Retroviral gene transfer—conclusion

Despite the limitations and safety concerns referred to above, retroviral-mediated gene therapy has already been used in a number of gene therapy attempts, and appears to hold a good deal of promise in this regard. However, although retroviral-mediated gene therapy has been used successfully for (nonhuman) germline modifications, the most used—and to date the most useful—method for germline transgenesis is microinjection, reviewed in the following section.

3.2. Microinjection

Jon Gordon in 1980 demonstrated that exogenous DNA could be introduced into the genome simply by the physical injection of a solution of cloned DNA into the zygote (Gordon et al., 1980). Subsequently, microinjection has become the most widely used method of germline transgenesis. The technique is most established with mice, however microinjection is also carried out fairly commonly with other animals including rats, rabbits, farmyard animals and fish.

A finely drawn out glass micropipette, loaded with DNA solution, is used for the injection. Under the microscope, with the aid of micromanipulators the egg is held fast and penetrated with the micropipette. The micropipette is guided through the cytoplasm towards one of the egg's pronuclei. Once the tip of the micropipette has entered the pronucleus, around 1–2 pl of DNA solution is injected, bringing typically 200 DNA molecules into the pronucleus. Some eggs lyse following microinjection, probably due to the physical trauma of being penetrated by the micropipette. Surviving eggs are transferred to the uterus of a pseudopregnant mother. Following gestation, between 10 and 40% of the resulting offspring are likely to be transgenic (Hogan et al., 1994).

In this method of transgenesis, the transgene DNA integrates into the endogenous DNA. Integration is random and usually occurs at only one chromosomal site in each transgenic. The number of copies of the transgene at an integration site may range from one to thousands. For multiple copy inserts, the most common arrangement is an array of molecules joined head-to-tail (reviewed by Gordon and Ruddle, 1985). Less usually, tail-to-tail and head-to-head arrangements occur. Deletions, duplications and other rearrangements may occur at the junctions between chromosomal and transgenic DNA sequences (reviewed by Bishop, 1996). Only a limited amount is known about the mechanisms of transgene integration.

It has become apparent that the majority ($\approx$ 85%) of pronuclear microinjection-derived transgenic founders are mosaics of transgenic and nontransgenic cells (Whitelaw et al., 1993). This surprising finding may be explained by postulating that (endogenous) DNA replication is required for chromosomal integration. If transgene integration occurs during DNA replication, only one of the two resulting cells will contain transgene sequences. During embryogenesis, a small number of cells (≥ 3 for mice) are recruited as embryo progenitors. Thus, the resulting animal will most frequently comprise a mixture of transgene-harbouring and transgene-free cells. In approximately 15–25% of mosaic founders, the foreign DNA apparently integrates at later stages of the embryo cell replication, resulting in mice that contain the transgene in varyingly small proportions of their cells.

As with other mosaics, transmission of the transgene is dependent upon the existence and extent of germline colonisation by transgene-containing cells. In the vast majority of cases where transmission occurs (whether from fully transgenic

founders, from mosaic founders or from subsequent generations of transgenics), the transgene is inherited in a stable mendelian fashion, although exceptions have been found (for example, see Palmiter et al., 1984). Due to the hemizygous nature of transgene insertion, even a nonmosaic founder will transmit its transgene to only (on average) 50% of its offspring.

3.2.1. Transgene design

There is no particular restriction on the size of DNA molecule used for microinjection. Yeast artificial chromosome (YAC) based transgene constructs consisting of >100 kb of DNA have been successfully introduced into the mouse germline by pronuclear microinjection (reviewed by Lamb and Gearhart, 1995). Indeed, it may become possible (pending development of the necessary transgene constructs) to microinject autonomous artificial 'mini-chromosomes' (mammalian artificial chromosomes, or MACs), complete with centromeres, telomeres and replication origins in addition to structural genes, promoters and enhancers. These specialized constructs would be expected to give a number of benefits compared with integrated transgenes, the most important of which would probably be the absence of chromosomal position effects on transgenic expression (reviewed by Sgaramella and Eridani, 1996). Because there are no special problems with microinjecting large transgene constructs, it is possible to incorporate structural gene-plus-promoter (plus other potentially useful sequences such as enhancers) combinations into the host genome. The main areas of application for microinjected gene-plus-promoter combinations (i.e. current transgene designs) are reviewed in the following sections. As noted previously, some of the fundamentals of transgene design also apply to other methods of transgene delivery, as should become clear in later sections of this paper.

3.2.2. 'Housekeeping gene' promoters

'Housekeeping gene' promoters, such as the β-actin promoter (Beddington et al., 1989) and the histone H4 promoter (Choi et al., 1991), can be fused with chosen structural genes. The 'housekeeping gene' promoters in such genetic constructs generally drive a fairly high level of constant transcription in most cell types and developmental stages when these constructs are integrated as transgenes.

3.2.3. Regulatory promoters

Beyond simply driving gene expression, promoters may be chosen to allow specificity in, or control over, patterns of expression. A transgene comprising a particular structural gene fused with a tissue-specific promoter should only produce its gene product in the tissue(s) specified by that promoter. In terms of germline gene therapy, this might allow treatment to be directed exclusively to the required tissues or organs. Another application would be to direct expression of a novel exogenous gene to a body part where the gene product will not cause physiological havoc, and where the product could be readily recovered. Indeed, pharmaceutically-useful products such as human factor IX and human alpha-1-antitrypsin (hAAT) have already been obtained from the milk of transgenic animals. Factor IX is an anti-haemophilic agent, and hAAT may be used for the treatment of emphysema. In the former case, the construct used comprised the factor IX structural gene fused with the ovine β-lactoglobulin promoter (Clark et al., 1989). The same promoter was used in the latter case, fused with the hAAT structural gene (Simons et al., 1987).

3.2.4. Control of transgene expression

If outside (i.e. experimenter) manipulation of gene expression is required, an inducible promoter may be used. Inducible promoters are able to respond to specific environmental cues such as temperature, or to dietary factors such as zinc. Thus the structural gene within a transgene can be switched on or off at will. For instance, a metallothionein (MT) promoter fused with a growth hormone (GH) gene (Palmiter et al., 1982) should allow GH production to be switched on simply by providing the transgenic with a zinc-supplemented diet. This might avoid the possible physiological difficulties associated with continuous global production—particularly in utero—of transgene products such as GH. Potential applica-

tions for inducible promoters in terms of gene therapy are conceivable.

More recently, inducible systems employing prokaryotic tetracycline resistance gene components have been developed (Gossen et al., 1995; Kistner et al., 1996; Park and Rajbhandary, 1998; Schultze et al., 1996; Shockett et al., 1995). These systems usually require two separate transgenes: thus, for use with transgenic animals (as opposed to cells in vitro) these systems usually require the establishment of two separate transgenic lines, each line containing one of the two transgenes. Double heterozygotes (containing both transgenes) are obtained by mating the two lines. One transgene (Transgene I) includes a promoter construct consisting of (a) an array of *tet* operator sequences and (b) a minimal promoter sequence; (a) and (b) are coupled to the gene that is to be controlled (Gene W). The other transgene (Transgene II) comprises a hybrid transcriptional transactivator gene fused to a suitable (e.g. tissue-specific) promoter. The hybrid transactivator gene product consists of a viral transcription-activating domain coupled with a tetracyline-binding domain. There are two main variants of the basic system: an 'on' system and an 'off' system. These variants are based upon functionally different transactivators. In the 'on' system, in cells in which Transgene II is active, exogenously administered tetracycline (or its analogue doxycycline) binds to the transactivator protein: this renders the transactivator able to bind to the *tet* sequences on Transgene I, thereby activating expression of Gene W. By contrast, the transactivator in the 'off' system binds to the *tet* sequences only in the absence of tetracycline: thus, administration of tetracycline prevents expression of Gene W.

Several other promoter-based systems for the control of transgene expression are in the developmental stage. Promising areas include natural promoters inducible by aryl hydrocarbons and promoter constructs inducible by steroid hormones (Fussenegger, 2001; No et al., 1996; Saez et al., 1997; Smith et al., 1995; Wang et al., 1994, 1997).

Site-specific recombination provides a novel means of controlling transgene expression (Kuhn et al., 1995; Stark et al., 1992; Utomo et al., 1999). As with the tetracycline approach (above), two separate trangenes are usually required, necessitating the mating together of separate transgenic lines to produce double heterozygotes (where transgenic animals are required). One transgene (Transgene I) consists of an appropriate (e.g. tissue-specific) promoter coupled to the gene that is to be controlled (Gene X), engineered to contain a strong stop signal flanked on each side by a recombinase recognition site (e.g. *loxP* from bacteriophage P1). The other transgene (Transgene II) consists of a recombinase gene (*Cre* in the case of bacteriophage P1) fused to an inducible promoter. Exogenous administration of inducer drives the production of Cre recombinase from Transgene II. The Cre recombinase binds to the *loxP* sites on Transgene I and catalyses the excision of the flanked stop signal, thereby rendering Gene X competent for expression. A variant of this system can be used to *inactivate* a transgene, in which Gene X (or an essential component thereof) is flanked by recombinase recognition sites. In this case, recombinase production results in the removal of essential sequences, thereby eliminating expression of Gene X.

3.2.5. Avoiding aberrant transgene expression

Transgene sequences integrating randomly into the host genome tend to give poor levels of expression, or exhibit inappropriate expression, in the form of temporally or spacially (ectopic) aberrant expression. The primary reason for such problems is the 'position effect', whereby the particular genetic environment at any point of insertion is likely to influence the expression of the integrated transgene. In some cases the remedy lies with transgene design: for example by ensuring that an appropriate enhancer sequence is included in the transgene construct. Beyond this, it may be possible to insulate a gene from the position effect. Matrix attachment regions are sequences which, when placed on either side of a gene within a transgene construct, appear to allow the gene(s) within an integrated transgene to occupy a separate chromosomal domain. Ultimately, however, the best solution to transgene expression problems would be to avoid the position effect entirely. This

is achievable through gene targeting: a transgene targeted to a chosen genomic locus will by definition avoid the position effect. As discussed in Section 2.2 and Section 2.3, reliable ways of germline gene targeting do exist. However, current germline gene targeting approaches rely upon the use of ES cells or NT, and gene targeting is not possible at present with pronuclear microinjection, due to the inability to select for eggs that contain rare targeted integrations (see Section 2.1). Therefore, the use of germline gene targeting as a means to avoiding transgene expression problems awaits progress in ES cell technology and NT technology.

3.2.6. *'Knockout' applications*

The above sections review ways in which gene function can be *added-in* by gene transfer. However, potential applications also exist for the ability to eliminate endogenous gene function in the recipient organism. For example, a dominant, gain-of-function single-gene disorder requires for its treatment the removal of the aberrant gene activity (rather than the addition of a missing activity). In animal transgenesis, the ability to eliminate specific gene function is often very desirable in the production of animal models for scientific/medical research.

Ideally, exogenous DNA would be targeted to the precise chromosomal locus in question. Integration of the transgene would cause disruption of endogenous DNA sequences, resulting in nonexpression of the endogenous gene. However, as discussed above, gene targeting is not widely available and is presently incompatible with pronuclear microinjection.

Until gene targeting becomes readily available, alternative methods of gene function elimination may be employed. One such approach is to fuse a toxin gene (e.g. the gene for diphtheria toxin A) to a particular promoter (for examples, see Palmiter et al., 1987; Wallace et al., 1991, 1994; Delaney et al., 1996). Thus, the cell population(s) in which the promoter is active will die (or simply fail to develop) due to intracellular toxin production.

An alternative approach employs a viral thymidine kinase (tk) gene as the expressing part of the transgene. Gancyclovir (GCV), a toxic thymidine analogue, may be administered systemically to the transgenic animal or, in principle, to the gene therapy-treated patient. GCV is relatively nontoxic unless phosphorylated by the viral tk gene product. Phosphorylated GCV is incorporated into the cellular genome during DNA replication, leading to cell death. Thus, dividing cells containing and expressing the transgene will be ablated. As with the 'toxin' transgenes considered above, promoter choice can determine which (transgene-containing) cells should express the tk gene. The tk/GCV approach is very promising for gene therapy applications, particularly in respect of antitumour approaches. Two properties are important in this respect: (a) the ability to 'switch on' transgene activity (only) when required, by administration of GCV; and (b) the ablation only of dividing cells.

Ablating entire cell populations is a rather heavy-handed way to eliminate specific functions. A more precise approach employs antisense DNA (reviewed by Sokol and Murray, 1996). This approach is feasible where the sequence of the gene to be turned off is known accurately. The antisense DNA represents the sequence of the non-transcribed DNA strand of the endogenous gene; the transgene construct is prepared by inverting the isolated structural gene and rejoining it with its promoter. Expression of the integrated transgene results in antisense RNA transcript production. The antisense RNA should hybridise with the 'sense' transcripts produced by the endogenous gene (located at a separate chromosomal locus). The resulting duplexes of RNA cannot be processed by the translational machinery, therefore protein production (hence, endogenous gene expression) is eliminated.

Beyond uses in basic research, antisense DNA constructs may have potential uses in gene therapy. An anti-viral strategy represents perhaps the most promising approach. DNA encoding an antisense viral RNA sequence would be coupled to an appropriate promoter to form the transgene construct. Once integrated into the host genome, the transgene should block viral replication by production of antisense RNA. An alternative potential application for antisense constructs might be in antitumour therapy.

Antisense DNA-based constructs do not, however, hold a great deal of promise for the treatment of dominant, gain-of-function single-gene disorders. The problem is that, unless the RNA sequence and size of a disease allele transcript happens to be quite different from that of the normal allele (assuming the sufferer to be a heterozygote), the antisense RNA may also act against the normal allele's transcripts.

3.2.7. Constraints on microinjection

Although microinjection is well established as an effective technique of transgenesis, it does have certain inherent limitations. Such limitations are reviewed in the following sections.

3.2.7.1. Practical constraints. The most obvious drawback of microinjection is its cost. Although conceptually very simple, the physical introduction of DNA into egg pronuclei requires sophisticated levels of equipment and expertise. Very high quality microscopes are required, together with micromanipulators, incubators, micropipette pullers, microinstrument forges, and a whole host of ancillary equipment. In terms of expertise it typically takes several years for a microinjector to be trained to a satisfactory level of competence. Further, microinjection requires such a high level of concentration over the long duration of a single session that it is usually impossible for an individual microinjector to perform efficiently more than about once per week. A single murine microinjection session lasts around 6 h. This (unbroken) period is dictated by the biological 'window' available between fertilisation and pronuclear fusion.

Added to the above costs is the problem of low absolute efficiency of transgenic production. For mice, a successful laboratory typically microinjects around 200 eggs per session. From these eggs, around 20 offspring should result, of which between 2 and 8 are likely to be transgenic. Thus the highest overall efficiency of murine microinjection is in the region of 4% (Hogan et al., 1994). Efficiencies for nonmurine mammalian species (e.g. cattle, pigs, sheep) are much lower, typically less than 1% (Wall et al., 1992). Such lower efficiencies may be accounted for by the fact that more expertise exists in respect of mice, and the existence of certain biological factors that hamper microinjection of nonmurine eggs.

A major limiting biological factor associated with cow, pig and sheep eggs is the difficulty of visualising their pronuclei. Cow and pig eggs are optically opaque, due to the presence of extensive cytoplasmic lipid particles. Sheep eggs do not have dense cytoplasmic particles, however their pronuclei remain extremely difficult to visualise. This effect seems to be due to the pronuclei and cytoplasm sharing similar refractive indexes.

Another difficulty concerns the size of pronuclei. Murine pronuclei are relatively large entities compared with those of farm animals. Thus, the micropipette must travel further into the farm animal egg compared with that of the mouse. The extra distance travelled by the micropipette causes a larger entry hole (due to the tapering section of the micropipette), thus increasing the likelihood of cell death. More difficulties with agricultural animal eggs include poorly anchored pronuclei and a lack of visible indicators of post-injection egg damage. For practical considerations concerning transgenesis by pronuclear microinjection, the reader is referred to the following excellent reviews: Wall (1996), Niemann and Kues (2000), Wolf et al. (2000).

Gene therapy via pronuclear microinjection has not been attempted with humans. Current knowledge of human gametes lends support to the idea that, for microinjection purposes, the human egg would probably be more akin to an agricultural animal egg than to the mouse egg.

3.2.7.2. Fundamental constraints. More basic than issues of cost and efficiency are limitations associated with the mode of transgene integration into the chromosome. The problem is that the whole process is, as mentioned previously, entirely random. There is no way of predicting or controlling major aspects of transgene integration such as copy number, copy orientation, endogenous sequence rearrangement nor, most importantly, chromosomal site of insertion (reviewed by Jaenisch, 1988).

As mentioned previously (in the context of retroviral vectors), each chromosomal locus im-

parts a particular position effect on the transgene. Thus, random integration may result in transcriptional failure in some transgenics, and always requires that each founder transgenic be treated as unique.

Another problem arising from random integration is that the exogenous DNA can disrupt and therefore inactivate an endogenous gene (Doerfler et al., 1997). Such new mutations are thought to occur in a small but significant number of transgenics.

In terms of applying transgenesis to particular purposes, it is not possible to deliberately introduce a nul mutation in zygotes using microinjection. Nor, therefore, is it currently possible to replace a particular gene function with a new function. The same applies to attempts to subtly alter (rather than switch-off) endogenous gene activity.

However, one speculative possibility might be to co-inject a recombinase enzyme (or gene) along with the transgene, in order to increase the frequency of HR, thus increasing the frequency of gene targeting. However, although several candidate enzymes/genes for enhancing HR have been described, the use of these in gene targeting is at an early experimental stage (Vasquez et al., 2001).

3.2.8. Non-zygote microinjection

Finally, it should be noted that microinjection can be applied to many types of cells in addition to zygotes.

ES cells fall into this category. For ES cells used in transgenesis, exogenous DNA must enter the cells somehow, and microinjection into the ES cell's nucleus is an effective option (see for example Zimmer and Gruss, 1989). The drawback is that microinjection does not allow ES cells to be treated en masse.

Somatic cells, whether in culture, temporarily removed from the body or, in principle, in situ in the body, may also receive exogenous DNA via microinjection (reviewed by Celis, 1984). As for ES cells, the drawback is the inability to treat cells en masse, together with enormous practical difficulties surrounding any attempt at in situ microinjection. (i.e. the huge numbers of cells likely to need microinjection and the difficulty of locating, visualising and manipulating such cells.) Thus, microinjection is fundamentally unsuitable for in vivo somatic cell gene therapy.

Somatic gene therapy via microinjection remains a possibility where ex vivo cells are involved. Microinjecting a relatively small number of ex vivo stem cells might, following their return to the body, allow the stem cells to recolonise and hence, amplify the number of treated cells. Alternatively, but more speculatively, somatic cells from foetal tissues could be microinjected prior to in vitro culturing; the treated cells would thus be amplified in culture, such that an adequate number could be (heterologously) transplanted to the patient.

Lastly, cells in culture may be microinjected simply in order to study the mechanisms of transgene integration.

3.3. Other methods of delivering transgenes

Retroviral infection and, especially, microinjection have become the main methods of gene transfer in higher animals, the former for somatic approaches and the latter for germline approaches. However, alternative methods are available or have been proposed. The following sections review a range of such methods.

3.3.1. Co-precipitation

Insoluble molecules such as calcium phosphate and DEAE-dextran can, when mixed with DNA molecules, co-precipitate to form granules. These granules are phagocytosed by cells and, in a proportion of recipients, the DNA appears in the nucleus where it may be transiently expressed. In a small proportion of treated cells, the DNA becomes stably integrated into the genome (Robins et al., 1981).

Despite its proven worth (and continuing use) with cultured somatic cells, co-precipitation is of limited use in transgenesis. Co-precipitation is somewhat laborious compared with electroporation and liposome-mediated gene transfer. There are no reports of successful gene transfer to eggs by co-precipitation. ES cells can be transfected by co-precipitation (Gossler et al., 1986), as can ex

vivo somatic cells. However, the range of potential cell types is limited: co-precipitation works well with fibroblasts, but has proved difficult to apply to other cell types. Even in non-refractory cell types, co-precipitation remains less than ideal because it is associated with transgene mutations and ultra-high copy number integrations (Calos et al., 1983; Razzaque et al., 1983). Additionally, for unknown reasons, co-precipitation appears to allow only relatively low levels of HR (reviewed by Mohn and Koller, 1995), making it less than ideal for gene targeting applications. Finally, co-precipitation is strictly an in vitro procedure: it is difficult to envisage ways in which it could be adapted for in vivo gene therapy.

3.3.2. Viral Infection

Retroviruses offer good opportunities for gene transfer due to their integration into the host chromosome. However, only actively diving cells are infectable by retroviral vectors, yet many potential targets for gene therapy are organs and tissues comprising slowly- or non-dividing cells. Thus, alternative viruses have been explored as potential agents for somatic cell gene therapy. Such viruses include the adenoviruses (Tomanin, 1997), hepatitis delta virus (HDV) (Netter et al., 1993) and herpes viruses (HSV) (Burton et al., 2001).

A major limitation with these viruses as transgene vectors is that it is unclear whether they can reliably be induced to infect ES cells, or eggs from a variety of species. Thus, at present the major potential application for these viruses lies in the domain of (somatic) gene therapy. However, given that most if not all cultured cell types can be made permissive for infection by such viruses, one might speculate as to the likelihood of ES cells being made similarly permissive by empirical advances. Alternatively, NT (Section 2.3) may in the future prove able to circumvent this limitation entirely.

3.3.2.1. Adenoviruses. Adenoviruses (Ad) have a number of properties that make them potential candidates for gene therapy. Ad are able to carry large transgenes (up to c. 38 kb) without adversely affecting their infectivity (Bett et al., 1993). Ad have a low host cell/species-specificity, providing a very large range of tissues and organs as putative candidates for treatment, and permitting current animal models to be used for testing adenovirus-mediated therapies. Transgene expression is stable and persists beyond 1 year after a single treatment (Stratford-Perricaudet et al., 1992). Moreover, given that: (a) serious disease following adenoviral infection is very rare; and (b) the viral genome rarely integrates into the host's chromosomes, adenoviral-mediated gene therapy promises higher safety levels than those associated with retroviral-mediated therapies (Lee et al., 1995).

In addition to their potential role in gene therapy applications, there are indications that Ad may be able to be used as transgene vectors. Tsukui et al. (1996) report the production of transgenic mice following the infection of zona-free eggs with a replication-defective Ad vector. This intruiging result suggests the possibility of a promising new strategy for animal transgenesis. However, further research is required in order to determine (a) the parameters within which Ad can reliably deliver transgenes to eggs; and (b) the range of species potentially amenable to this form of gene transfer.

3.3.2.2. Hepatitis delta virus. HDV is potentially usable as an agent of gene therapy directed at somatic cells. HDV is self-replicating, and may reach high copy-numbers ($\approx 300\,000$ per cell) in infected tissues. This property makes HDV attractive as a gene therapy vector, although there are safety issues to be considered in view of the possible consequences of germ cell infection by a self-replicating vector. Another safety consideration is that HDV is potentially cytopathic, although it might be possible to modify the viral genome to reduce such effects. A limitation with HDV is its size: at only 1.7 kb, it is unlikely to be able to carry large exogenes (Netter et al., 1993).

3.3.2.3. Herpes viruses. HSV have large (125–229 kb) genomes, and therefore offer the potential for transferring large exogenes (Roizman, 1994). Herpes viruses also have the attractive capacity of being able to induce permanent latent infection in their hosts (Stevens, 1989). Although herpes viruses certainly offer great potential for gene

therapy, particularly in respect of disorders of the nervous system (reviewed by Lachmann and Efstathiou, 1997), the development of gene therapy systems employing these viruses awaits substantial progress in certain areas, including the ability to control the viral life-cycle and to prevent immune attack of treated cells. Further, the pathology of herpes viral infection is poorly understood; thus there remain potential safety problems to be resolved.

3.3.2.4. Other viruses. The viruses considered above are not the only types of viruses that are under scrutiny as transgene vectors. No single virus has the necessary characteristics for all applications. Nor have all possible types of viruses been assessed for the potential utility in somatic or germline transgenesis. Other types of viruses will doubtless be added to the current store of potential gene transfer vectors.

3.3.3. Liposome-mediated gene transfer

Liposomes are artificial vesicles that can act as delivery agents for exogenous materials including transgenes (see excellent reviews by Watwe and Bellare, 1995; Nicolau et al., 1987; Ilies and Balaban, 2001). Like their natural cellular counterparts, liposomes comprise a lipid bilayer surrounding a small volume of aqueous solution. The liposome's lipid bilayer is similar to that of natural cells, consisting mainly of phospholipids and cholesterol. Liposomes have been created in a variety of distinct forms, some of which are available as commercial preparations: the major differences are of structure, size and charge. The main structural difference is between liposomes with a single lipid bilayer and those with a multilamellar ('onion-skin') lipid bilayer. Liposome sizes range from around 100 nm to several micrometers, and may be either negatively or positively charged.

Liposomes for use as gene transfer vehicles are prepared by adding an appropriate mix of bilayer constituents to an aqueous solution of DNA molecules. In this aqueous environment, phospholipid hydrophilic heads associate with water while hydrophobic tails self-associate to exclude water from within the lipid bilayer. This self-organising process creates discrete spheres of continuous lipid bilayer membrane enveloping a small quantity of DNA solution. The liposomes are then ready to be added to target cells (Felgner, 1996; Mahato et al., 1997).

When they come into contact with a target cell, liposomes may interact with the cell membrane in a variety of ways. Possible liposome–cell interactions include: (a) exchange of membrane constituents (Lipsky and Pagano, 1985); (b) adsorption to the cell membrane (Eggens et al., 1989); (c) endocytosis (Connor et al., 1984); and (d) membrane fusion (Lamb, 1993). Interactions (a) and (b) are undesirable, as they result in the transgene molecules remaining outside the cytoplasm. Interaction (c) is undesirable where it results in the formation of late endosomes, as the outcome is the destruction of engulfed transgene molecules. Nevertheless, interaction (c) may be desirable where early endosomes release transgene molecules into the cytosol. Interaction (d) is desirable because liposome–cell fusion allows the exogenous DNA to directly enter the cytosol. 'Simple' liposome systems comprise negatively or neutrally charged liposomes: interaction (d) is quite rare in such systems, and generally takes several days for completion (Schaeferridder et al., 1982). Although the success rates (with cultured cells) are better than those obtained using standard co-precipitation gene transfer protocols, the relative rarity of liposome–cell membrane fusion presents a problem for transgenesis and gene therapy per se. Furthermore, the slow rate of fusion is particularly problematic for in vivo therapies because free liposomes are rapidly captured and destroyed by macrophages (Schoilew et al., 1990).

More recent systems employ cationic liposomes. The positively charged lipids in such liposomes bind directly with the cell membrane. Such liposomes have been developed into commercially available systems that offer ease of preparation, stability and high transfection efficacy: examples include: Lipofectin™, LipofectAMINE™, and Transfectace™ (Gibco-BRL); Transfectam® (Promega); and DOTAP (Boehringer Mannheim) (reviewed by Chisholm, 1995).

An alternative approach to the problem of inefficient liposome–cell membrane fusion in-

volves the use of fusion-inducing virus glycoproteins. Glycoproteins from several viruses (including parainfluenza viruses, paramyxoviruses, coronaviruses and retroviruses) have powerful cell-fusion promoting properties, and inclusion of virus glycoproteins responsible for these properties into the liposome lipid bilayer results in a more frequent and rapid occurrence of liposome-cell fusion (Bailey and Chernomordik, 1997).

The major potential use of liposomes is in gene therapy, particularly with in vivo somatic approaches, for example in antitumour approaches (Dass et al., 1997). In this context, liposomes rival viral vectors as a DNA delivery method. Given that they consist only of biological lipids, liposomes have low toxicity. There is a theoretical risk for patients with lipid metabolism disorders; however studies designed to assess this have suggested minimal actual risk (for example see Tsuboniwa et al., 2001). Antigenicity does not appear to be a problem either (Yanagihara et al., 1995), and there are of course no concerns about viral proliferation within the host.

Cationic liposome-mediated gene therapy has been carried out using animals/animal models. For example, partial correction of the ion transport deficit in the cystic fibrosis mouse model was reported following instillation (Hyde et al., 2000) and nebulization (Stern et al., 1998) of liposomes. Similarly, Canonico et al. (1994) and Losordo et al. (1998) have reported expression of hAAT and GH genes in rabbits following aerosol delivery and intravenous injection of liposomes.

Fusion-inducing glycoprotein-based liposomes have been used to deliver genes in vivo in several animal studies. Gene expression has been reported following injection of such liposomes into various tissues including heart, kidney, liver, lung, skeletal muscle and testis; gene expression has also been reported following trans-arterial delivery (reviewed by Yanagihara et al., 1995).

Although liposome-based gene transfer systems are now able to efficiently deliver transgenes to the cytoplasm, only in a minority of cells will transgenes reach the nucleus. This represents a significant limitation in terms of transgene stability and expression. One way of avoiding this problem might be to characterise and utilise the biochemical apparatus that permits some viruses (e.g. poxviruses: Carroll and Moss, 1997; Moss, 1996) to exist and replicate in the cytoplasm.

Following liposome-mediated gene transfer, amongst transgene molecules reaching the nucleus, only a minority integrate into the host cell chromosomes. Transgene expression is therefore essentially transient, with reported durations of expression varying widely between separate studies: the range is from around 10 days to several months, with a typical duration of perhaps around 20 days (see reviews by Ilies and Balaban, 2001; Yanagihara et al., 1995).

For in vivo gene therapy, transient expression would necessitate repeated administration of liposomes, perhaps for the entire lifetime of the patient. However, this may not be a fundamental problem, given the previously mentioned low toxicity and antigenicity associated with liposomes, although a suitably non-invasive administration route would certainly be required. From a safety perspective, the non-integration that underlies transient expression would be positively beneficial for in vivo therapies, particularly in respect of oncogenesis but also with regard to the genetic integrity of the patients' germ cells.

Germline transgenesis is possible with liposome-mediated gene transfer, and ES cells have been successfully transfected by liposomes (for example see Pain et al., 1999). However, the rate of transgene integration into the genome is broadly similar to that of electroporation. As with electroporation, the relatively low integration frequency renders liposome-mediated transfection impractical for use with mammalian zygotes.

However, an indirect approach to the germline via the zygote remains a putative possibility, where liposomes would be used to deliver transgenes to sperm cells. Some success has been claimed for this approach (reviewed by Smith, 1999).

In summary, liposomes look set to become increasingly important as agents of transgene delivery, particularly for in vivo gene therapy. Efforts are being exerted towards improving and developing methods in a number of respects, including the following areas (Sections 3.3.3.1, 3.3.3.2 and 3.3.3.3).

3.3.3.1. Improved site-specificity of in vivo treatment. Beyond simply changing the site/route of liposome administration, accurate organ targeting might be achieved by incorporating tissue-specific recognition molecules (e.g. receptor-binding proteins, antibodies) into the liposome membrane (Tiukinhov et al., 1999).

3.3.3.2. Improved nuclear targeting. Efficient translocation of exogenous DNA to the host cell's nucleus might be achieved by incorporating nuclear-localisation proteins into the liposome complex (reviewed by Boulikas, 1997). Improved nuclear translocation should: (a) enhance expression levels; and (b) increase the frequency of chromosomal integration, thus increasing the scope for germline modification.

3.3.3.3. Improved expression longevity. If chromosomal integration could be induced (see previous section), expression could persist for the lifetime of the host cell; however, nuclear translocation without integration would be very useful in itself. The capacity of liposomes to carry DNA molecules of great size (more than 150 kb has been reported—see for example Strauss et al., 1993) may in future allow transfer of MACs. Long-lived expression would be an expected outcome.

3.3.4. Electroporation

Electroporation is a process by which high-intensity electric field pulses temporarily destabilise cellular membranes. During the destabilisation period, DNA molecules present in the surrounding media are able to permeate the cell's external and internal membranes to enter the cytoplasm and nucleoplasm (reviewed by Lurquin, 1997).

Electroporation provides a fast and inexpensive means of introducing exogenous DNA into cultured mammalian cells. Electroporation is not associated with transgene mutation. The process can be equilibrated to yield copy numbers (of integrated transgenes) of between 1 and 20 copies per genome—an advantage compared with microinjection. Large transgene molecules ($\geqslant$150 kb) can be transferred. In addition to the advantages of being able to transfer large conventional transgenes, the DNA transfer capabilities of electroporation may in future allow transfer of MACs (Section 3.2.1). Such constructs should be immune to nuclease attack and might be designed to replicate in step with the host cell cycle, thus providing long-lived transgene expression. The main drawbacks of electroporation are that: (a) specialised equipment is required; (b) each cell type and culture system requires fairly extensive empirical optimisation; and (c) typically only around 0.01% of treated cells show genomic integration of transgene (see excellent reviews by Chang et al., 1992; Lurquin, 1997; Potter and Cooke, 1992).

In terms of mammalian transgenesis, electroporation is an effective method of introducing exogenous DNA into ES cells (Chu et al., 1987). The advantage of electroporation over microinjection in the context of ES cells is that electroporation allows the en masse treatment of large numbers of cells. This is extremely useful where a rare integration event requires selection from a background of unwanted integrations, as in gene targeting. Similarly, electroporation has been successful with NT transgenesis (Mccreath et al., 2000; Schnieke et al., 1997).

The relatively low efficiency of electroporation renders it impractical for use with mammalian zygotes. The best superovulation protocols deliver around 30 eggs per animal for mice and pigs, and 10 for cattle and sheep (Wall et al., 1992). Extrapolating from these figures, a 0.01% efficiency rate would necessitate on average 300+ mice or pigs, and approximately 1000 cattle or sheep in order to obtain just one transgenic. However, for (mammalian) species that produce large numbers of easily recovered eggs, electroporation shows more promise in terms of transgenesis. Many fish species are potentially useful in this respect, and some successes have been claimed. For example, Murakami et al. (1995) report the successful use of electroporation to create transgenic medaka, as do Ono et al. (1997), with the latter also reporting successful transmission of transgenes to F_1 progeny.

An attractive putative use of electroporation for transgenesis would be as an adjunct to sperm cell-mediated DNA. For example, Gagne et al. (1991) report an increase from 12 to 19% of transgenic bovine blastocysts when electroporation is in-

cluded in an otherwise passive sperm-DNA uptake protocol. Similar findings were reported by Rieth et al. (2000), again with transgenic bovine blastocysts. Several experiments have indicated that fish species may be able to be genetically manipulated in this way (for examples see Walker et al., 1995; Patil and Hong Woo, 1996). However, these results await replication, and big questions remain over the effectiveness or otherwise of sperm cells as vectors per se (Smith, 1999).

If it turns out that there is substance to claims that sperm cells can be induced to carry transgenes, then the techniques' efficiency would have to be high. Otherwise, electroporation of sperm cells could be as limited as it is (in principle) for zygotes, with excessively high numbers of animals needed in order to obtain each transgenic. The only way around this limitation would be via the development of a selection system for 'positive' sperm cells in vitro—a highly unlikely possibility, give that sperm cells exist in a quiescent state as far as gene expression is concerned.

In terms of its use in gene therapy, electroporation has the potential to be used in vivo. This field is at a very early stage of development, but empirical improvements may in future permit electroporation to be used to deliver transgenes to particular tissues or to tumours (Hofmann et al., 1999; Swartz et al., 2001). Clearly, this has great potential utility in the context of gene therapy. Moreover, given that there is no evidence that any particular somatic cell types are inherently unable to be successfully electroporated, the range of treatable tissues—and hence, diseases—is potentially very great.

Ex vivo gene targeting approaches using electroporation are also under development. Hatada et al. (2000) used electroporation to correct by gene targeting a defective hypoxanthine phosphoribosyltransferase gene in hematopoietic progenitor cells. The approach was similar to gene targeting with ES cells or NT, in that selection was used to enrich for targeted outcomes. If similar successes can be obtained with appropriate stem cells in humans, it may be possible to return such targeted cells to the body of the patient such that repopulation by the altered cells yields a therapeutic or curative outcome for various genetic disorders.

Finally, electroporation has recently, been used to transfer genes into cultured mammalian embryos at defined stages of development (Akamatsu et al., 1999; Osumi and Inoue, 2001). The purpose here is to gain insights into mammalian development at the molecular level (as opposed to generating transgenic animals). In the experiments described by Osumi and Inoue (2001), plasmid DNA was injected into the neural tube of rat embryos prior to electroporation. Following in vitro maintenance of the electroporated embryos, exogenous DNA was detected in 10–100% of the cells in the target region.

3.3.5. *Particle bombardment*

Finally, it is worth considering a highly novel technique, as an illustration of the many and varied means by which emerging technologies are enabling gene transfer. In particle bombardment, DNA may be adsorbed onto spherical tungsten or gold particles (diameter c. 4 μm) and transferred into a mass of cells by a particle gun; once inside the target cells, the DNA is solubilised and may be expressed (Klein et al., 1992). This approach, sometimes known as 'biolistics', was originally developed for plant transgenesis but has been shown to be effective for transferring transgenes into mammalian cells in vivo (Cheng et al., 1993). Indeed, there are indications that biolistics may be more efficient than alternative methods such as liposome-mediated transfection and recombinant viral infection (Gainer et al., 1996), although the amount of research data presently available is too little to permit definitive comparisons. If the method does prove to be effective in vivo, tumours are the most likely targets for particle bombardment (reviewed by Mahvi et al., 1997).

Biolistics then, is a promising method for treating cells en masse, and looks most useful in terms of somatic gene therapy. There have been no reported attempts to utilise biolistics for altering germline cells. The en masse nature of the approach places it in a similar position to that of electroporation or liposome-based methods in respect of fertilised eggs: impractically large numbers of eggs would undoubtedly be required per successful transgenic event. In principle it might be possible to apply biolistics to ES cells as a route to

the germline, however to date no such attempts have been recorded.

4. Concluding remarks

The ability to transfer genes into the higher animal cells is a prerequisite for continued progress in animal transgenesis and in human gene therapy. However, improvements in gene transfer are urgently required, particularly if hopes of effective gene therapies are to be realised. Key aspects for attention include improved control of target cell range, improved transgene uptake efficiencies, the ability to localise transgenes to the nucleus and improvements in gene targeting to enable the efficient integration of transgenes into chosen genomic loci.

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